ends that were compatible with *Cla*I-restricted DNA.

*Sth*134I is a potentially useful R-ENase with commercial feasilibity, because it could be isolated from the food-grade organism *S. thermophilus* and it has a higher optimal temperature than most type II R-ENases.

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Isolation and characterization of a type II restriction endonuclease from *Streptococcus thermophilus*

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1. SUMMARY

A type II restriction endonuclease *Sth*134I was isolated from *Streptococcus thermophilus* strain 134. The enzyme is an isoschizomer of *Hpa*II. The restriction endonuclease is most active at Mg(II) $\geqslant$ 5 mM; in the pH range of 7.5–8; temperature of 50°–55°C; and (KCl) or (NaCl) below 100 mM. Double digestion and ligation experiments showed that *Sth*134I apparently recognized and cleaves DNA at the sequence $C^{1}CGG$ to produce two-base, 5′-protruding ends.

2. INTRODUCTION

Streptococcus thermophilus is an essential microorganism in the production of many types of fermented dairy foods, where its main function is the synthesis of lactic acid and other flavor compounds. Interest in its molecular genetics and its use as a host expression system for foreign genes has been steadily increasing [1–4]. This is undoubtedly based on the food-grade status of this microbe permitting its addition to human food.

Direct introduction of plasmid DNA into *S. thermophilus* has become possible recently with the development of an electrotransformation procedure in our laboratory [5]. In those studies, we noted the recalcitrance of several strains of *S. thermophilus* to electrotransformation with plasmid DNA. This led us to screen for the presence of restriction endonuclease (R-ENase) activity that may influence the survival of transforming DNA in these organisms. This report describes the first type II R-ENase that has been characterized in *S. thermophilus*.

3. MATERIALS AND METHODS

3.1. Organism and growth condition

Streptococcus thermophilus strain 134 was from our laboratory culture collection. The bacteria were maintained in lactose broth as previously described [6].

3.2. Isolation of Sth*134I*

Bacterial cultures (800 ml) were grown at 37°C for 16 h without agitation. DL-threonine at 20 mM was included in the medium to facilitate subse-

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quent cell breakage [7]. Cells were harvested by centrifugation (5 000 × *g*; 10 min; 4 °C). The pellet was washed once in 1/10 volume of TGMB buffer (10 mM Tris-HCl, pH 7.6; 10% glycerol; 20 mM MgCl; 20 mM 2-mercaptoethanol containing 0.2 mM phenylmethylsulfonylfluoride (PMSF), and resuspended in the same buffer at 1/50 to 1/25 the original culture volume. Cell breakage was effected by sonication (Sonicator Model W-225; Heat Systems- Ultrasonics Inc., Farmingdale, NY) for 1–2 min at 4 °C. Cell debris was removed by centrifugation (11 000 × *g*; 30–45 min; 4 °C), yielding a supernatant which constituted the crude extracts. R-ENase in the supernatant was salted-out by adding saturated $(NH_4)_2SO_4$ aqueous solution (pH 7) to a final 60% saturation. After ⩾ 30 min on ice, the precipitate was collected by centrifugation (11 000 × *g*; 1 h; 4 °C) and dissolved in ⩾ 5 ml TGMB buffer. Anion exchange chromatography was performed on a 1.5 cm × 7 cm DE-52 (Whatman Ltd., Maidstone, Kent, U.K.) column equilibrated with TGMB buffer. After sample application, the column was washed with TGMB until A_{280nm} of the eluate returned to baseline. Elution was with 70 ml of a KCl linear gradient (OM → 0.3 M KCl) in TGMB. This was followed by elution with TGMB + 0.3 M KCl until A_{280nm} again reached the baseline value.

Fractions with R-ENase activity were pooled, concentrated, and equilibrated in TGMB using a Centricon-30 microconcentrator (Amicon, Danvers, MA). The enzyme preparation was stored as liquid at 4 °C, in frozen state at −20 °C, or in TGMB containing 50% glycerol at −20 °C. The R-ENase appeared to remain active for at least 3 months under these storage conditions.

3.3. *Restriction, ligation, and analysis of DNA*

PhiX174 RF DNA (Form I), SV40 viral DNA (Form I), pBR322 and pUC19 plasmids were purchased from BRL Life Technologies, Inc. (Gaithersburg, MD), as were the restriction endonucleases *Hpa*II and *Cla*I. Plasmid pVA736 was prepared from *Streptococcus sanguis* Challis according to a procedure described previously [8]. DNA digests were analysed by submerged, horizontal agarose gel (SeaKem ME, FMC BioProducts, Rockland, ME) electrophoresis in TBE (89 mM Tris-borate; 89 mM boric acid; 2 mM Na_2EDTA, pH 8) buffer system [9]. *Hin*dIII-fragments of Lambda DNA (Sigma Chemical Co., St. Louis, MO) were used as size markers

Unless otherwise specified, *Sth*134I activity was assayed by incubating the sample with 0.2–0.4 μg PhiX174 RF DNA for 0.5–1.5 h at 50 °C or 55 °C. Column fractions were first dialyzed versus TGMB to remove excess salt. For relatively crude samples, the reaction mixture was extracted with phenol-chloroform-isoamyl alcohol mixture [9]. When necessary, DNA was precipitated in Na-acetate and ethanol solution [9].

DNA fragments were dephosphorylated and ligated by using calf intestinal alkaline phosphatase (CIP, Boehringer Mannheim Biochemicals, Indianapolis, IN) and T4 DNA ligase (BRL Life Technologies, Inc., Gaithersburg, MD), respectively [10].

4. RESULTS AND DISCUSSION

4.1. *Isolation of* Sth*134I*

Cell breakage by sonication for 1–2 min was most suitable for the release of a type II R-ENase activity from *S. thermophilus* strain 134. The $(NH_4)_2SO_4$ precipitation step effectively separated the majority of DNases from the type II R-ENase. The R-ENase activity salted out from crude extracts at 50–60% $(NH_4)_2SO_4$-saturation; the general nucleases, on the other hand, were precipitated at ⩾ 80% saturation of $(NH_4)_2SO_4$.

Anion exchange chromatography removed the undesired nucleases and proteins from the R-ENase. Fig. 1 shows that the type II endonuclease, which cleaved hiX174 RF DNA into two prominant fragments of 2.8 and 1.7 kbp and other smaller but discrete pieces (⩾ 0.5 kbp), was eluted from the column at between 30 mM and 80 mM (KCl). Nonspecific nucleases, on the other hand, desorbed from the column only at (KCl) > 125 mM; the substrate was digested by these fractions to a spectrum of fragments without discrete sizes. The chromatogram also shows that the bulk of contaminating proteins as indicated by A_{280nm}, was effectively separated from the type II R-EN-

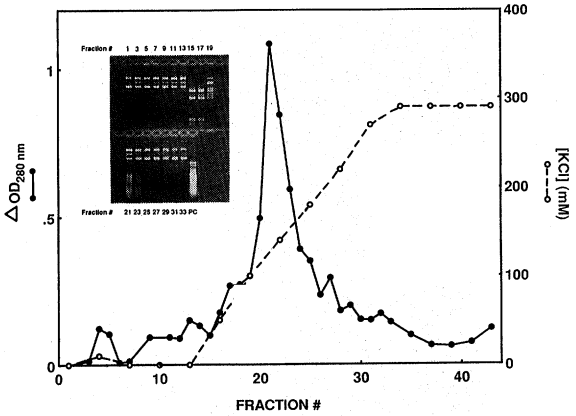

Fig. 1. Isolation of *Sth*134I by DE52 Anion Exchange Chromatography. Column: 1.5×7.0 cm; flow rate: 0.5 ml/min; fraction volume: 3.4 ml (KCl) was determined by conductivity measurement. Inset: R-ENase Assay of Column Fractions. PhiX174 RF DNA (0.8 μg) was mixed with 50 μl of fraction and incubated at 50°C for 1 h. Reaction mixtures were phenol-extracted and ethanol-concentrated as described in text. Electrophoresis was performed on a 1% agarose gel. PC, sample prior to column chromatography.

ase activity. According to convention [11], we named this R-ENase as *Sth*134I.

4.2. *Optimal reaction conditions of* Sth*134I*

Reaction conditions were optimized with PhiX174 as substrate. As with all known type II restriction enzymes, *Sth*134I had an absolute requirement for Mg(II). Fig. 2 shows that enzyme activity was observed only at $[MgCl_2] \geq 5$ mM. Closer examination of the gel picture revealed that fluorescent bands representing products of partial digestion were less intense at higher $[MgCl_2]$, suggesting that stronger enzyme activity was attained at higher Mg(II). The optimum temperature range for *Sth*134I was 50°–55°C. Enzyme activity was only slightly lower between 32° and 45°C, but was dramatically reduced at temperatures ≥ 60°C. A pH dependence study showed that *Sth*134I exhibited strong activity in the pH range of 7.0 to 8.3, with an optimum occurring at 7.5; only negligible enzyme activity was observed at pH ≥ 6.5. Activity of *Sth*134I was reduced in the presence of 100 mM NaCl or KCl, but complete inhibition was not attained even at 200 mM salt concentration. The high optimal temperature and relative insensitivity to NaCl or KCl of *Sth*134I distinguished it from its well-known isoschizomer *Hpa*II (see below).

4.3. *Cleavage site of* Sth*134I*

Figs. 1 and 2 show that PhiX174 was cleaved by *Sth*134I into five major fragments of 2.8, 1.7, 0.4, 0.35 and 0.2 kbp in sizes, along with other partial digests. SV40 viral DNA (Form I) was linearized by the enzyme; and pBR322, pUC19 and Lambda DNA's were each restricted into numerous fragments (data not shown). Comparing these observations with those from previously reported R-ENases of Streptococci [12], *Sth*134I appeared to be an isoschizomer of *Sfa*GUI and *Hpa*II. In agreement with this assignment, *Sth*134I cleaves pVA736 plasmid into two fragments (6.9 and 0.7 kbp) corresponding in size to those ob-

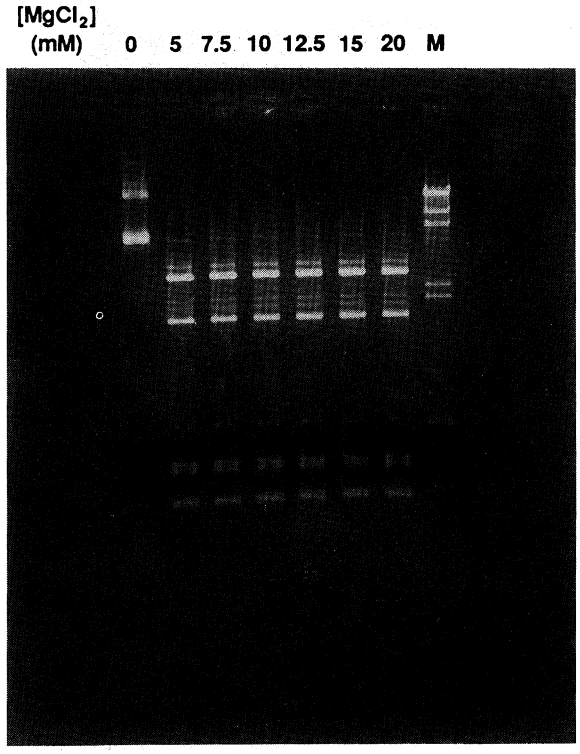

Fig. 2. Mg(II) Dependent of *Sth*134I Activity. DE52-purified *Sth*134I and PhiX174 RF DNA (0.4 μg) were incubated at 55°C for 30 min in 9 mM Tris-HCl (pH 7.6)+4.5 mM 2-ME at indicated ($MgCl_2$). Agarose gel concentration was 1%. M, *Hin*dIII-digested lambda DNA markers (0.8 μg).

tained with *Hpa*II digestion [13]. The isoschizomeric relationship of *Sth*134I and *Hpa*II was confirmed by results of a double digestion experiment, shown in Fig. 3. When PhiX174 was digested either sequentially (lanes 5 and 6) or simultaneously (lanes 7 and 8) with *Sth*134I and *Hpa*II, the DNA fragments produced were all accounted for in single digestion with *Sth*1341 (lanes 1 and 2) or *Hpa*II (lanes 3 and 4). If they were not isoschizomers, the double digestion reactions would have yielded additional DNA fragments that were not produced in single digestion reactions.

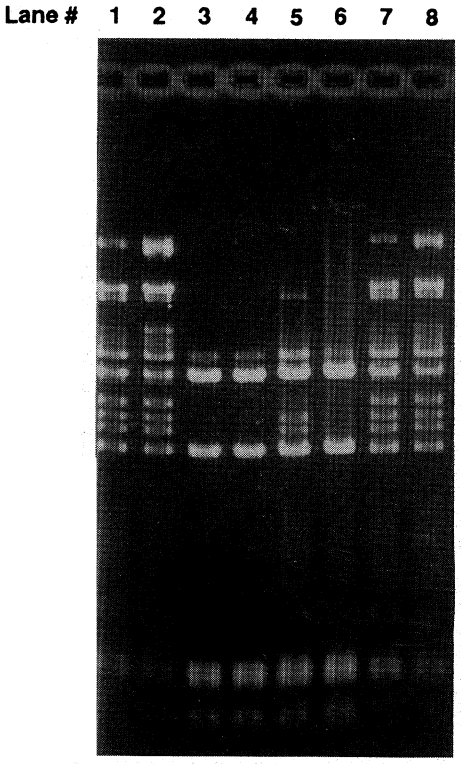

Fig. 3. Double Digestion of PhiX174 RF DNA by *Sth*134I and *Hpa*II. *Sth*134I activity obtained from 60% $(NH_4)_2SO_4$ precipitation was used. Substrate PhiX174 DNA was at 0.8 μg. Lanes: 1 and 2, *Sth*134I, 50 °C and 37 °C for 1 h, respectively; 3 and 4, *Hpa*II, 50 °C and 37 °C, 1 h, respectively; 5, *Sth*134I, 50 °C, 1 h, followed by *Hpa*II, 37 °C, 1 h; 6, *Hpa*II, 37 °C, 1 h, then *Sth*134I, 50 °C, 1 h; 7, *Sth*134I + *Hpa*II, 50 °C, 1 h; 8, *Sth*134I + *Hpa*II, 37 °C, 1 h. Electrophoresis was performed on a 0.8% agarose gel.

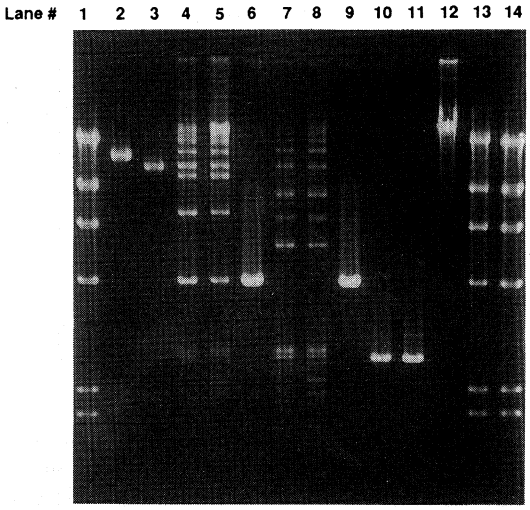

Fig. 4. Ligation of *Sth*134I- or *Hpa*II-generated PhiX174 Fragment (2.8 kbp) and *Cla*I-linearized, CIP-treated pBR322. Lanes: 1, 13, and 14, *Hin*dIII-digested lambda DNA markers (0.5 μg); 2 and 3, 14.3-kbp and 11.6-kbp DNA markers, respectively; 4, 0.25 μg *Sth*134I-PhiX174 (2.8-kbp fragment) + 0.4 μg *Cla*I-pBR322 + T4 DNA ligase (1.5 μ); 5, 0.5 μg *Hpa*II-PhiX174 (2.8-kbp fragment) + 0.4 μg *Cla*I-pBR322 + ligase; 6, 0.4 μg *Cla*I-pBR322 + ligase; 7, 0.25 μ *Sth*134I-PhiX174 (2.8 kbp) + ligase; 8, 0.3 μg *Hpa*-PhiX174 (2.8-kbp) + ligase; 9, 0.4 μg *Cla*I-pBR322; 10, 0.25 μg *Sth*134I-PhiX174 (2.8 kbp) 11, 0.3 μg *Hpa*II-PhiX174 (2.8 kbp); 12, 0.5 μg lambda DNA *Hind*II-fragments + ligase. Electrophoresis was on a 0.7% agarose gel.

As with *Hpa*II, DNA cleavage by *Sth*134I yielded a two-base, 5′-protruding ends with the sequence of CG. Results in Fig. 4 show that when 2.8-kbp *Sth*134I-fragment of PhiX174 was ligated with *Cla*I-linearized, CIP-treated pBR322 DNA, successful ligation was achieved, yielding a host of ligation products (lane 4). Among these products was a 7.2-kbp DNA that represented the 1 : 1 ligation between the two reactants, signifying the absolute compatibility of the ends generated by *Cla*I and *Sth*134I. As expected, when 2.8-kbp *Hpa*II-fragment of PhiX174 was used in the reaction, identical ligation products (lane 5) were obtained as with the *Sth*134I fragment. In both instances, the product profiles were noticeably different from the self ligation of the 2.8-kbp fragments (lanes 7 and 8). These results conclusively showed that similawr to *Hpa*II, *Sth*134I cleavage of DNA resulted in 5′-CG protruding